CHAPTER

10 Molecular Diagnostics

contents

I. INTRODUCTION

A. Genetic Information

1. All genetic information in procaryotic and eucaryotic cells is contained in **deoxyribonucleic acid** (DNA) sequences, which are arranged as genes and packaged into chromosomes.
2. Human genome: **3 billion base pairs**
3. Estimated number of genes in human genome is less than 25,000
4. The genetic information is transferred from parent to daughter cells by DNA **replication.**

B. Central Dogma

1. Central dogma describes the transfer of genetic information within a cell. DNA is used as a template for **ribonucleic acid** (RNA) synthesis: **DNA → RNA → Proteins.**
2. The process of **DNA → RNA** is termed **transcription.** One strand of DNA is copied into messenger RNA (mRNA) by RNA polymerase II.
3. The process of **RNA → Protein** is termed **translation.** A molecule of mRNA is read by ribosomal machinery in the cytoplasm, resulting in the production of proteins that perform cellular functions.

II. NUCLEIC ACIDS

A. Basic Structure of Nucleic Acids

1. Nucleic acid is a polynucleotide, a linear polymer of nucleotides, made up of three components: nitrogenous bases, 5-carbon sugars, and phosphate groups.
   a. **Nitrogenous heterocyclic base** (purines and pyrimidines) is attached to the 1' carbon atom of the sugar by an *N*-glycosidic bond.
      1) **Purines** have a double ring and include adenine (A) and guanine (G).
      2) **Pyrimidines** have a single ring and include cytosine C, thymine (T), and uracil (U).
      3) Both DNA and RNA contain A, G, and C. T is only found in DNA, and U is only found in RNA.
   b. Cyclic 5-carbon sugar residue
      1) RNA contains a ribose sugar, which has OH groups bound to its 2' and 5' carbons.
      2) DNA contains a deoxyribose sugar. Deoxyribose is identical to ribose except that the OH group at the 2' position has been replaced with a H atom.
   c. A phosphate group is attached to the 5' carbon of the sugar by a phosphoester bond, and it is responsible for the strong **negative charge** of both nucleotides and nucleic acids.
2. The nucleotides are joined to one another by a second phosphoester bond between the 5' phosphate of one nucleotide and the 3' OH group of the adjacent nucleotide. This arrangement is called a **phosphodiester bond.**

B. Physical and Chemical Structure of DNA

1. **Base pairing**
   a. Base composition of DNA varies from organism to organism; however, A always pairs with T (or U in RNA) and G always pairs with C. This is termed **complementary base pairing.** In DNA, the number of adenines equals the number of thymidines, and the number of guanines equals the number of cytosines.
   b. **In a double-stranded DNA molecule, the concentration of purines always equals the concentrations of pyrimidines.**
2. **Structure**
   a. Watson and Crick (1953) described DNA as two polynucleotide strands coiled about one another to form a **double-stranded helix.**
   b. **Sugar-phosphate backbones** of each strand form the outer edge of the molecule, and bases are in the central core.
   c. Each base in one strand is hydrogen bonded to a complementary base in the other strand, which forms the purine-pyrimidine base pair (bp) (e.g., AT and GC). Two hydrogen bonds form between A and T, whereas three hydrogen bonds form between G and C.
   d. At physiological temperatures, the DNA base pairs are stable; however, they can break and reform rapidly.
   e. A DNA helix has two external grooves: the **major groove,** where various protein molecules bind to DNA, and the **minor groove.**
3. **Complementary strands**
   a. Due to base pairing rules, the two strands are complementary. For example, if one strand has the sequence 5' GATACC 3', the other strand's sequence is its complement: 3' CTATGG 5'. See below.
   5' G–C 3'
   A–T
   T–A
   A–T
   C–G
   3' C–G 5'.
   b. The two strands of the DNA double helix are **antiparallel;** their chemical orientations are different.
      1) As diagrammed above, one strand runs in the 5' to 3' direction, whereas the opposite strand goes from 3' to 5'. The 3' OH end of one strand is opposite the 5' P end of the other.
      2) By convention, a base sequence is usually written with the 5' P terminal end on the left.
4. **Eucaryote chromosomes**
   a. **Eucaryotes** have multiple chromosomes.
      1) Eucaryotes have a nucleus surrounded by a nuclear membrane. The chromosomes are contained within the nucleus.

2) Helical human DNA is compacted into chromosomes and bound to basic proteins called histones. DNA and a histone complex is called **chromatin.**
3) The human nucleus contains **46 chromosomes.**
   a) Humans have two copies of each chromosome, a condition called **diploid.** There are two types of chromosomes: somatic and sex.
   b) **Somatic chromosomes:** 22 pairs, numbered 1 to 22
   c) **Sex chromosomes:** X or Y, XX = female and XY = male

b. **Eucaryote genes**
1) Coding parts of a gene are called **exons** (**ex**pressed sequences). They are well conserved; the nucleotide sequence does not vary significantly among individuals of the same species.
2) Noncoding regions of a gene are called **introns** (**in**tervening sequences). They can contain regulatory/transcriptional elements and have other functions.
3) Approximately 25% of all human genes have multiple allelic forms called **polymorphisms. Allele** refers to a different version or form of a gene or noncoding region. For example, the human leukocyte antigen **(HLA)** locus, which codes for peptides that establish self-identity of the immune system, is highly polymorphic. At these **loci** single nucleotide changes occur frequently. Loci are the physical locations or positions of a gene or noncoding region on a chromosome.

c. **Procaryotic chromosome**
1) Procaryotes lack a nucleus and nuclear membrane.
2) The chromosome is generally circular DNA with groups of related genes arranged in a linear fashion. Approximately 95% of the chromosome contains coding sequences and 5% is noncoding sequences.

d. **Plasmids** are extrachromosomal DNA containing nonessential genetic information. In certain situations, plasmids give organisms a growth advantage. For example, resistance (R) plasmids contain genes that confer antimicrobial resistance to a bacterium. Plasmids replicate independently of the chromosome in cells and can be passed from one generation to the next.

5. **Human mitochondrial DNA (mtDNA)**
a. Circular genome of approximately 16,600 base pairs, inherited maternally
b. mtDNA contains 22 tRNA genes, 2 rRNA genes, and 12 genes coding for oxidation-phosphorylation components. Mutations in these genes are responsible for neuropathies and myopathies.
c. Noncoding regions (610 bp): Hypervariable regions I (342 bp) and II (268 bp) are routinely sequenced for forensics.

C. Three-Dimensional Structure of DNA and RNA

1. Native conformation of DNA is double stranded (ds); whereas the disrupted form, known as **denatured** (or melted) DNA, is single stranded (ss).

Denaturation can be accomplished through **heating** or **chemicals.** Although denaturing temperatures for DNA vary by species, assays requiring denaturation of human DNA are conducted at about 94°C.

2. **Renaturation,** or **annealing,** is the association of denatured DNA to native dsDNA.
   a. Many molecular biology techniques are based on the reassociation of complementary base sequences (i.e., **hybridization**) and can be used to:
      1) Determine whether certain sequences occur more than once in DNA of a particular organism
      2) Locate specific base sequences in a DNA molecule
      3) Detect particular type of RNA
   b. There are two requirements for annealing:
      1) Salt concentration must be high enough to overcome electrostatic repulsion between negatively charged phosphate groups in two strands.
      2) Temperature must be high enough to disrupt the random, nonspecific intrastrand hydrogen bonds. Annealing of human DNA will occur around 52°C.

D. DNA Replication

1. DNA replication is a process in which genetic information is transferred from parent to daughter cells. It requires energy to unwind the helix and disrupt H-bonds.
2. **Proofreading and repair systems** exist to minimize replication errors; however, mistakes do sometimes occur and on occasion can be expressed as mutant phenotypes.
   a. Base changes occur resulting in **mutations;** sometimes these mutations do not have an effect, producing **polymorphisms.**
   b. Often mutations have detrimental effects. However, some mutations result in a selective advantage for the organism that is the basis for evolution.
3. The synthesis of each nucleotide chain only occurs in the 5' → 3' direction. One strand is synthesized continuously, whereas the other strand is synthesized discontinuously, resulting in **Okazaki fragments** that must be ligated together by the enzyme ligase.
4. Each parental DNA strand serves as a template to create a complementary daughter strand.
   a. As replication proceeds, the parental double helix unwinds by the action of enzymes called **helicase** in procaryotes and **topoisomerase** in eucaryotes.
   b. Polymerization of DNA is catalyzed by enzymes called **DNA polymerases.**
   c. As a new strand is formed, it is hydrogen bonded to its parental template. Each new double helix consists of one parental strand and one newly formed daughter strand.

E. RNA Overview

1. Most RNA molecules are **single stranded;** however, RNA readily forms secondary structures.
2. Generally, RNA is environmentally labile and easily degraded.
3. **Types of RNA**
   a. **Ribosomal RNA (rRNA)** makes up 80–90% of total RNA in a cell; it is part of ribosomes and is involved in translation of mRNA into proteins.
   b. **Messenger RNA (mRNA)** makes up 2.5–5% of total RNA in cell; it is an intermediate between the genetic code in DNA and the protein product. mRNA is read by ribosomes to produce proteins.
      1) In eucaryotes, transcription of DNA forms a pre-mRNA molecule with both introns (noncoding) and exons (coding regions). This molecule is referred to as **heteronuclear RNA** (hnRNA). The introns are removed, and the exons are joined together.
      2) Further processing into mature mRNA includes addition of a 5' methylguanine cap and polyadenylate (poly A) tail of up to 200 adenylate nucleotides at the 3'-OH terminus.
   c. **Transfer RNA (tRNA)** reads mRNA triplets and brings the appropriate amino acid to the ribosome for polypeptide (i.e., protein) synthesis. There is at least one tRNA for each amino acid.
   d. Other RNAs: **Small nuclear RNA** (snRNA) is involved in removal of introns and **small and micro RNAs** (including siRNA, stRNA, miRNA, snoRNA) are involved in cellular processes.

III. NUCLEASES AND RESTRICTION ENZYMES

A. Definitions

1. A variety of enzymes called **nucleases** break phosphodiester bonds in nucleic acids (they usually exhibit chemical specificity).
2. **DNases:** Deoxyribonucleases
   a. Many act on either ss or dsDNA.
   b. Some act on both ss and dsDNA.
3. **RNases:** Ribonucleases
   a. Ubiquitous, very high concentrations on hands; thus it is necessary to wear gloves when working with RNA
   b. Act at a wide range of temperatures: below –20 to >100°C
4. **Exonucleases** cut only at the **end** of a nucleic acid, removing a single nucleotide at a time.

B. Mechanisms of Activity

1. **Restriction enzyme/endonucleases** recognize a specific base sequence in a DNA molecule and cut near or within the sequence. These enzymes make two cuts, one in each strand, generating a 3'-OH and a 5'-P terminus.

a. **Star activity** refers to nonspecific cleaving when incubation conditions are not optimal.
b. Several classes of restriction enzymes are known.
c. **Type II restriction enzymes make cuts at predictable sites within or near the recognition sequence;** they have the greatest utility in recombinant DNA experiments.
    1) Sequences recognized by most **type II** enzymes are known as **palindromes,** which are sequences with **bilateral symmetry.**
        a) The **sequence reads the same** from the **5' to 3' direction** on **both strands.**
        b) The enzyme binds the specific sequence and cleaves the DNA directly at the binding site to produce fragments of predictable size.
        c) For example, the enzyme *Eco*RI recognizes the sequence 5' GAATTC 3' and cuts between the G and A. On the complementary strand, the sequence reads 3' CTTAAG 5' and is cut between A and G.
    2) A particular restriction enzyme generates a unique **family of fragments** for a particular DNA molecule. A different enzyme generates a different family of fragments from the same DNA molecule.
    3) In human DNA, occurrence of restriction enzyme sites are polymorphic, so that the pattern of DNA fragments produced by a particular restriction enzyme digest may differ from person to person.
        a) Analysis of **restriction fragment length polymorphisms** (RFLPs) has been used to screen and diagnose hereditary diseases.
        b) RFLPs can be used in **forensics and criminology.** Southern blot analysis using probes to detect short, inherited hypervariable sequences produces a characteristic RFLP for each individual and thus produces a unique "genetic fingerprint."
    4) DNA fragments can be visualized via fluorescent stains in electrophoresis systems using agarose or polyacrylamide gel or fused silica capillary tubes filled with polymer. Examples of fluorescent stains are ethidium bromide and SYBR green.

IV. LABORATORY TECHNIQUES IN MOLECULAR DIAGNOSTICS

A. Nucleic Acid Isolation

1. **Sources**
    a. A variety of clinical specimens can be used: body and lavage fluids, saliva, buccal cells, stool filtrate, bone marrow, and whole blood. **Nucleated cells** (e.g., white blood cells) are needed for DNA isolation.
    b. Tissue: Fresh, frozen, paraffin embedded (must dewax with xylene or other agents, then rehydrate)
    c. **Mitochondrial DNA:** Usually obtained from hair follicles
    d. Approximately 1 million eucaryotic cells, 10–50 mg of tissue, yield about 10 μg RNA.

e. **Microorganisms:** Bacteria must be treated with enzymes and detergents that destroy their cell walls in order to release nucleic acids.

2. **Special considerations for RNA isolation** to avoid degradation of RNA
   a. Because hands have high concentration of RNase, **gloves should always be worn.**
   b. Use equipment dedicated to RNA testing.
   c. RNase-free reagents must be used.
   d. Reserve areas in the laboratory for storage of reagents and RNA work.
   e. Use disposable items direct from manufacturer.
   f. Avoid reusable glassware or **bake 4–6 hours at >270°C** to inactivate RNase.
   g. Lyse cells using detergent, or phenol, in presence of high salt or **RNase inhibitors** (e.g., **guanidine thiocyanate**) and a strong reducing agent, such as 2-beta-mercaptoethanol.
3. **Isolation methods**
   a. **Organic isolation**
      1) High-salt, low-pH, and organic mixture of phenol and chloroform to remove contaminating proteins, lipids, carbohydrates and cellular debris (for RNA isolation, use acidic phenol pH 4–5)
      2) DNA and RNA collected in aqueous phase
      3) DNA or RNA is **precipitated** using **ethanol** or **isopropanol** in high-salt concentration and recovered as pellet by centrifugation.
      4) The pellet is rinsed with 70% ethanol and the DNA resuspended in 10 mM Tris, 1mM EDTA (TE), or sterile DNase-free water and RNA in RNase-free buffer/water.
   b. **Inorganic isolation**
      1) Low pH and high salt concentration are used to precipitate protein leaving DNA in solution.
      2) DNA is precipitated using isopropanol and resuspended as previously noted.
   c. **Solid phase isolation**
      1) Silica-based products bind DNA or RNA in high-salt concentration.
      2) Cells are lysed and nucleic acids released and placed in high-salt buffer. The sample is added to a column, allowing the nucleic acids to absorb to the matrix. The column is washed to remove impurities, and the nucleic acids are eluted with low-salt buffer.
      3) Examples of automated systems for DNA extraction: Roche MagnaPure® and Qiagen BioRobot®
   d. **Chelex extraction**
      1) Chelex is a cation-chelating resin used in forensic applications.
      2) A solution of 10% Chelex is mixed with the specimen. The mixture is boiled. The contaminating substances will bind to resin and are removed by centrifugation.
      3) DNA will be in the supernatant.

e. **Isolation of poly-(A)-enriched RNA (mRNA)**
   1) The poly-A tail of mRNA binds to oligomers of poly-T or poly-U immobilized to a **matrix** (i.e., beads or resin).
   2) Total RNA lysate is poured over the matrix. Poly-A RNA binds poly-T or -U resin; matrix is rinsed and then mRNA is eluted in low-salt buffer with detergent.
   3) Generally, 1 μg total RNA yields 30–40 ng poly-(A)-enriched RNA.

4. **Measurement of quality and quantity of isolated nucleic acids**
   a. **Electrophoresis:** DNA and RNA are anionic (negatively charged).
      1) **Genomic DNA** is intact and, therefore, has a high molecular weight.
      2) **Restricted DNA:** Genomic DNA is treated with restriction enzymes, creating fragments that can be separated according to size.
      3) RNA is electrophoresed in the presence of **chaotropic agents** to prevent degradation. A chaotropic agent, or chaotrope, is a substance that disrupts the three-dimensional structure of macromolecules. In total RNA preparation, intact 28S and 16S rRNA bands should be present at about 4.8 and 1.7 kilobases.
      4) Semiquantitative estimates of nucleic acid are made by comparison to ethidium bromide stained standards.
   b. **Spectrophotometry**
      1) **Absorptivity maximum for nucleic acids is near 260 nm.**
         a) 1 Absorbance (Abs) unit equals 50 μg/mL dsDNA. Therefore, the concentration of dsDNA can be determined by multiplying the absorbance reading by 50 and by the dilution factor of the sample.
         b) 1 Abs unit equals 40 μg/mL RNA or ssDNA. Therefore, the concentration of RNA or ssDNA can be determined by multiplying the absorbance reading by 40 and by the dilution factor of the sample.
      2) **Absorptivity maximum of phenol is 270 nm;** therefore, phenol contamination can give falsely high readings.
      3) **Absorptivity maximum of protein is 280 nm.**
      4) **Quality estimates:** Determined from the ratio of Abs 260 nm:Abs 280 nm
         a) For high-quality DNA, the ratio will be 1.6–2.0.
         b) For high-quality RNA, the ratio will be 2.0–2.3.
   c. **Fluorometry:** For dsDNA only, Hoechst 33258 dye binds minor groove of A-T base pairs

B. Nucleic Acid Sequence Identification by Hybridization

1. Identification of a DNA fragment in a sample that carries a particular gene or base sequence, called the **target sequence,** can be accomplished by denaturing the fragments and measuring the ability of the separated strands to renature (i.e., **hybridize**) with a **labeled** (ss) **probe** containing the same (i.e., **homologous**) sequence or part thereof.

2. Used in four main disciplines: microbiology, immunology, forensics, and genetics
3. Two types of hybridization methods: **in solution** and **solid membrane support.**
   a. With solution methods, both target and probe sequences are in a liquid state.
      1) Example: AccuProbe® from GenProbe, Inc.
      2) Detects **rRNA of organisms** using an **acridinium-labeled ssDNA probe**
      3) The **rRNA:DNA hybrid** protects the label from hydrolysis by selection reagent. Detection reagents are added, and **chemiluminescent signal** is read in a **luminometer.**
   b. **Blots:** Techniques in which target or probe sequences are immobilized on a solid matrix
      1) Target sequences (i.e., DNA, RNA, or proteins) in samples are blotted onto **solid membranes** made of **nitrocellulose, nylon,** or **polyvinyldifluoride.**
         a) **Southern blot:** Template molecules are DNA fragments produced by restriction enzyme digestion separated by gel electrophoresis, chemically denatured, and transferred to the membrane.
         b) **Northern blot:** After electrophoresis, total RNA or mRNA is transferred to a membrane.
         c) **Western blot:** Proteins are separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and then transferred to a membrane.
      2) **Transfer** of macromolecular template (i.e., DNA, RNA, or proteins) onto solid matrix can be accomplished by **capillary action, electrical current, pressure,** or **vacuum.**
      3) **Labeled probes of known sequence** are placed in liquid buffer and are incubated with the blot to hybridize with DNA or RNA sequences on the blot that are complementary to the labeled probe.
      4) After hybridization, the blot is washed to remove unbound probe, the solid support is allowed to dry, and the signals produced by bound probe:target hybrid sequences are measured.
      5) Factors that influence hybridization conditions **(stringency)** for probe binding (annealing) and removal of probe (washing) include temperature and salt and/or denaturant concentration in buffer.
         a) **Temperature**
            i) **Melting temperature** (T_m) is the temperature required to separate hybridized strands of complementary nucleotide sequences. At the T_m, half of the double-stranded structure has dissociated into single strands.
            ii) Calculate **annealing temperature** ($T_m - 5°C$) so that probes can hybridize to specific template.

iii) **High temperature:** High stringency; only exact (complementary) matched sequences in target and probe bind and remain bound
iv) **Low temperature:** Low stringency; some mismatched sequences may bind

b) **Salt concentration of buffer**
i) **Low salt concentration:** High stringency (distilled water is highly stringent liquid)
ii) **High salt concentration:** Low stringency

c) Presence of denaturant in buffer can help increase stringency, allowing only highly matched sequences to bind

c. **Dot blot/slot blot**
1) Cells are lysed, and the DNA is denatured and added to a membrane. A probe is added. After washing, the bound probe is detected.
2) Positive (with target sequence) and negative (without target sequence) control samples must be included on membrane.

d. In **macroarray** and **microarray** analyses, often termed **reverse hybridization,** a number of known sequences **(probes)** are spotted onto a solid membrane or a glass slide, microchip, or microelectrode. Nucleic acids from patient samples are labeled with a fluorescent dye in solution (using polymerase chain [PCR] technology) and incubated with the macroarray/microarray. Patient nucleic acid will bind to probes with complementary sequences and produce fluorescence in those spots.
1) Target and/or probes sequences can be **DNA, RNA, protein, complementary DNA (cDNA), PCR products, or synthesized oligonucleotides.**
2) **Structural analysis** of multiple genes, gene sequences, mutations, and polymorphisms can be accomplished by this method.
3) Starting with RNA, cDNA, or protein products obtained from patient samples allows **gene expression** analysis.

4. **Probes**
a. **Definition:** Single-stranded piece of DNA, RNA, or short oligomer whose base sequence is complementary to that of the target nucleic acid
b. dsDNA probes must be denatured, or made single stranded, by heating at 95°C or heating to 75°C in 50% formamide.
c. The purpose of a probe is to identify a sequence of interest within a large amount of nucleic acid.
d. Produced by synthesis of oligonucleotides or cloning
1) Short (<500) bases: Less specific, good for mutation analysis
2) 500–5000 bases: Greater specificity, less affected by mutations
3) Because of secondary structure formation, high GC content decreases efficiency of binding of probe to target.
e. Probes are labeled with **radioisotopes** (e.g., ^{32}P), **enzymes** (e.g., alkaline phosphatase or horseradish peroxidase), **fluorescent,** or **chemiluminescent** compounds to produce a measurable signal.

1) Multiple fluorescent dyes can be used concurrently to detect different targets in same reaction mixture, or chemiluminescent labels with different kinetic profiles can be employed (e.g., "flash" and "glow" signals.)
2) Incorporation of labels can be performed using **labeled primers** or a **labeled deoxynucleotide triphosphate (dNTP)** in a DNA synthesis reaction, such as PCR.

f. **Detection** methods include exposure to X-ray film, scintillation counting, fluorescent microscopy, or measurement by a fluorometer, colorimeter, spectrophotometer, or luminometer.

5. **Chromosomal structure and mutations**
   a. **Giemsa (G) banding** of metaphase spread
      1) **Karyotype:** The complete set of chromosomes in a cell
      2) Detect translocations, deletions, insertions, and copy number
   b. **Interphase fluorescence *in situ* hybridization (FISH)**
      1) The probe is a 60- to 200-kb fragment of DNA specific for a chromosomal region and covalently attached to fluorescent molecule.
      2) Interphase FISH detects chromosomal copy number, translocations, deletions, and amplified chromosomal regions in cells in interphase. A major advantage of this method over metaphase FISH is that it does not require culturing of cells.
   c. **Metaphase FISH** uses chromosomes in metaphase and probes that bind to part of or the whole chromosome (chromosome painting).

C. Uniqueness of DNA between Individuals

1. DNA is purified from samples. Specific regions or parts of genes are amplified by PCR to produce products (i.e., **amplicons,** products of natural or artificial amplification events) that are then treated with restriction enzymes. The resulting restriction fragment pattern is analyzed.
2. **Single nucleotide polymorphism (SNP or "snips")**
   a. The **Human Genome Project** revealed that SNPs occur as frequently as every 100–300 bases. The majority of these sequence differences are variations of single nucleotides or SNPs.
   b. Despite numerous polymorphisms, any two people are 99.9% identical at the DNA sequence level. A 0.1% difference accounts for disease susceptibility and other variations among "normal" human traits. In addition, about 80% of the 0.1% will be SNPs.
   c. Can be used for genetic mapping, disease prediction, disease associations, and human identification
3. **Variable number tandem repeats (VNTR)**
   a. Repeats of eight or more nucleotides
   b. Can be repeated 10–50 times in tandem, which can be detected by Southern blot or PCR
4. **Short tandem repeats (STR)**
   a. Repeats of 1–10 base sequences in tandem that can be detected by PCR

b. **Trinucleotide repeat expansion** seen in some genetic diseases (e.g., fragile X syndrome [>200 CGG repeats] and Huntington disease, or Huntington chorea [>37 CAG repeats])
c. STR typing in forensics now uses **tetranucleotide** (four bp repeat unit)
   1) Primers are designed to produce amplicons 100–400 bp in length.
   2) STR alleles are identified by PCR product size. Sizes of PCR products are determined by the number of embedded repeats.
   3) By having one of each of the primer pairs labeled with a fluorescent marker, the PCR product can be analyzed in fluorescent detection systems (e.g., **capillary electrophoresis**). The number of loci that can be resolved on a single run has been increased by the use of multicolor dye labels.
   4) STRs are present throughout the genome. Sets of multiplexed primers are used for **human identity testing.**
      a) **CODIS** (Combined DNA Indexing system) uses 13 "core" polymorphic loci and the nonpolymorphic amelogenin locus on the X and Y chromosomes.
      b) Two different sized amelogenin PCR products are seen in males (XY), whereas only one size amelogenin product is seen in females (XX).
      c) STR match is made by comparing profiles and calculating probability statistics as indicator of relatedness.

D. Amplification Methods

1. **Increase sensitivity** of test system by making more copies of **target,** or **probe,** or attaching more **signal** producing molecules onto target
2. **Target amplification methods:** PCR, RT-PCR, qPCR, and transcription based amplification
3. **Probe amplification methods:** Ligase chain reaction (LCR), strand displacement amplification (SDA), and QB replicase
4. **Signal amplification methods:** Branched chain DNA (bDNA) amplification, hybrid capture assay (HCA), and cleavage based amplification

E. Polymerase Chain Reaction

1. Prototype method used to exponentially increase the amount of target DNA found in a sample, making detection more sensitive
2. Over time has become more automated, allowing it to have more applications
3. Conventional **three-step cycle**
   a. Sample DNA is **denatured** by heating to **94–96°C,** 20–60 seconds.
   b. **Primers** are added and the sample cooled to **50–70°C,** 20–90 seconds to allow **primers to anneal** (i.e., bind complementary sequences).
   c. **DNA polymerase** (i.e., ***Taq* polymerase**) is added at **72°C** for 10–60 seconds to **extend primers** and complete **DNA synthesis** of target sequence defined by primers. *Taq* polymerase is a thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus*.

d. A **thermocycler** automatically changes temperatures and allows cycling to occur within a reaction vessel.
e. **Repeat the cycle** 20–30 times to produce detectable levels of amplicons.
f. After x cycles, a 100% yield $= 2^{x-2}$ (e.g., with 30 cycles, $2^{28} = 2.68 \times 10^8$ amplicons from one template). The yield is not exactly 2^x because the products from the first two cycles are slightly larger than the desired product.
g. Unlabeled products can be detected by gel electrophoresis or labels can be introduced into the product using labeled primers or dNTP. Alternatively, labeled probes or antibodies that recognize products that give off **colorimetric, fluorescent** or **chemiluminescent signals** can be used.

4. Components
   a. **Template or target nucleic acid** (100 ng–1 μg) in sample
   b. **Primers** are short oligonucleotide sequences that are complementary to the sequence flanking the 3' end of the target region of interest for each of the two DNA template strands.
   c. **Deoxynucleotide triphosphates** (i.e., dATP, dCTP, dGTP, and dTTP): Building blocks that extend primers to form PCR product (i.e., amplicon)
   d. **DNA polymerase,** such as *Taq* polymerase, extends the primers by adding dNTPs complementary to template to form PCR product.
5. **Controls in PCR testing**
   a. **Positive control:** Known sample containing target sequence
      1) Ensures that DNA polymerase enzyme is active
      2) Buffer is optimal
      3) Primers are annealing to correct sequence
      4) Thermocycler is working properly
   b. **Blank control or reagent control:** Reaction without DNA added to ensure that reagent mix is not contaminated with template or previously amplified PCR products
   c. **Negative template control:** DNA sample known to lack target to ensure that primers do not anneal to unintended sequence
   d. **Internal control or amplification control** is a second primer set for a sequence unrelated to target sequence of interest but present in all samples tested. It can be performed in same tube or can be run as a duplicate sample.
      1) Ensures that DNA sample does not contain inhibitors and reaction mix is working properly
      2) Distinguishes between true negative (sample without target sequence) and false negative (amplification failure) results
6. **Ways to avoid PCR contamination**
   a. Physical separation of areas for preparation of sample, reagents, PCR mixes, and amplification and post-amplification procedures
      1) Positive air pressure in preparation room, negative air pressure in PCR/post-PCR rooms, air locks, isolation cabinets, dedicated equipment, and gloves

2) Work areas should be decontaminated with ultra violet light, bleach, and alcohol.
3) Unidirectional organization of workflow; never go from post-amplification area to preparation areas

b. **Chemical: dUTP-UNG system** (AmpErase® system)
1) The system uses PCR mixtures with dUTP rather than TTP; therefore, amplicons contain uracil rather than thymidine residues.
2) Uracil-*N*-glycosylase (UNG) is added to the reaction mixture.
3) The mixture is incubated at 50°C for 2–10 minutes to eliminate any contaminating amplicons from previous reactions. Native DNA lacks uracil and is immune from degradation.
4) During the first denaturation step of the PCR cycle, UNG enzyme is destroyed and amplification occurs only if target sequence is found in sample.

F. Quantitative Real-Time PCR (qPCR)

1. Estimates the amount of starting template (e.g., copy number/mL)
2. Fluorescently labeled primers are incorporated into PCR, thus generating fluorescently labeled amplicons. Fluorescent signal is generated as target copy number increases during amplification process.
3. Analysis is done during the exponential phase of the reaction. The amount of fluorescence generated is directly proportional to amount of starting template; however, the time to its accumulation (the point when it crosses a predetermined amount or threshold) is inversely proportional, such that large amounts of target cross threshold early.
4. The cycle number at which fluorescence crosses a threshold is designated **C_T (threshold cycle). C_T is inversely proportional** to the **amount of starting target.** Thus, detection of a large amount of target is indicated by a lower C_T value.
5. **Applications:** Viral load, tumor load, and treatment monitoring
6. **Detection systems**
   a. **SYBR green** is a double-stranded DNA-binding dye that monitors accumulation of PCR products as they are made (i.e., in real time). Mispriming and/or primer dimers are artifacts that generate fluorescence.
   b. **Probe systems to increase specificity** have been developed and produce fluorescence only when hybridized to target sequences.
      1) **TaqMan probe:** Probe of ssDNA oligomer is homologous to a specific sequence in the targeted region of the PCR template. As PCR product is amplified, the probe is displaced and hydrolyzed, releasing fluorescence.
      2) **Molecular beacons** measure accumulation of product at the annealing step in the PCR cycle. Signal is detected only when probes are bound to template before displacement by polymerase.
      3) **Scorpion primer/probes** are tailed with hairpin molecular beacons structure. In the presence of template, primer/probe is extended moving

reporter molecule away from quencher molecule, which generates fluorescence.

4) **Fluorescent resonance energy transfer** (FRET) utilizes two probes: one with a 3' fluorophore and one with a 5' catalyst for the fluorescence that binds to adjacent target sites on the amplicon.

G. Reverse Transcription PCR (RT-PCR)

1. **RNA template** is converted to a DNA copy (cDNA) by RNA-dependent DNA polymerase; also known as **reverse transcriptase.**
2. **cDNA product** then serves as template to make millions of copies of target RNA sequence.

H. Transcription-Based Amplification Systems

1. Transcription-mediated amplification (TMA; GenProbe), nucleic acid sequence–based amplification (NASBA), and self-sustaining sequence replication are examples of **target amplification** methods.
2. **RNA is the target and primary product.**
3. **Reactions are isothermal.**
4. **Applications:** Direct detection of RNA viruses and RNA from other infectious agents, as well as transcribed gene sequences

I. Probe Amplification Methods

1. Ligase chain reaction (LCR) and strand displacement amplification (SDA) are commercially available in the U.S.; QB replicase is available in Europe.
2. Number of target sequences in sample is not changed. Synthetic primers/probes complementary to target nucleic acid are amplified.
3. **Ligase chain reaction**
   a. Entire target sequence must be known to design four oligonucleotide primers.
   b. LCR primers bind adjacent to each other, separated by only one base.
   c. **Enzymes: DNA polymerase** synthesizes cDNA by extending primers, and **DNA ligase** seals gap between adjacent primers.
   d. **Thermocycler needed**
   e. **Applications:** Detect point mutations in known genes (e.g., beta-globin gene for detection of sickle cell disease)
4. **Strand displacement amplification**
   a. Major amplification products are the probes/primers.
   b. Denaturation, then isothermal two-stage process of **target generation** followed by **exponential amplification phase**
   c. First stage involves **target generation;** primer and probe bind close to each other. Probes have recognition site for a **restriction enzyme (RE).**
      1) As the outer primers are extended, they displace the probes, which are extended.

2) A second set of complementary primers then binds to displaced probes, and **DNA polymerase** extends the complementary primers, producing a double-stranded version of the probes.
3) The probes are the target DNA for the next stage of the process.

d. Second stage: **Exponential probe/target amplification phase.**
1) When RE is added to ds probe DNA, only one strand of the probe will be cut, leaving a nick in the DNA that was extended. The opposite strand is simultaneously displaced. The displaced strand is copied by the primers.
2) Iterative process takes place at **52°C** without temperature cycling and produces millions of copies of the initial sequence.

e. Addition of fluorogenic probe yields a fluorescent signal directly proportional to the amount of amplified probe/target.
f. **Application:** SDA is the basis for the BD ProbeTec®ET for *Mycobacterium tuberculosis, Chlamydia trachomastis,* and *Neisseria gonorrhoeae*.

5. **QB replicase** is an RNA-dependent RNA polymerase from bacteriophage QB. The target can be either denatured DNA or RNA.
a. Probe-bound template is amplified by mixing with QB replicase, which can generate a billion RNA molecules/probes in less than 15 minutes.
b. Used for identification of infectious agents

J. Signal Amplification Methods

1. bDNA amplification, HCA, and cleavage-based amplification
2. In **signal amplification** systems, the number of target sequences does not change; instead, **large amounts of signal are bound to the target sequences present in the sample,** making detection more sensitive. These systems carry **less risk of target contamination.**
3. **bDNA** is frequently used for quantification of target sequences in clinical samples, especially **viral load** determinations.
a. A series of short oligomer **probes captures** target nucleic acids.
b. Additional **extender (or amplifier) probes** bind target nucleic acids, and then **multiple reporter molecules** load target nucleic acid with signal.
c. **Procedure:** (1) **Target** nucleic acid (either RNA or DNA) is released from cells; (2) DNA is **denatured;** (3) target nucleic acid binds to **capture probes,** fixed to solid support; and (4) **extender probes** have sequences that are complementary to sequences in the target molecules and to sequences in the **amplifier molecules.** Binding of complementary sequences occurs.
d. **Reporter molecules labeled with alkaline phosphatase** bind amplifer probes. **Dioxetane** is added as **substrate** for alkaline phosphatase, and a **chemiluminescent signal** is emitted.

4. **HCA** is marketed by Digene Diagnostics for detection of human papillomavirus, hepatitis B virus, and cytomegalovirus.
   a. **Target DNA** is released from cells and binds to **ssRNA probes** to form DNA/RNA hybrid molecules.
   b. **DNA/RNA hybrid** forms unique structure, which can be **bound by antibodies** to surface of microtiter well.
   c. **Captured hybrids** are detected by binding **alkaline phosphatase-conjugated anti-DNA/RNA hybrid antibodies** in a typical "sandwich" assay.
   d. Substrate is added and signal is measured.
5. **Cleavage-based amplification:** Based on activity of cleavase enzyme used in the **Invader® assay** (Third Wave Technologies, Inc.)
   a. Detects target nucleic acid by a series of probes that bind to the target and overlap
   b. **Cleavase** recognizes overlapping sequence of DNA and cuts it.
   c. During isothermal incubation, if probe and test sequences are complementary, two enzymatic cleavage reactions occur, resulting in a fluorescent signal.
   d. **Applications:** Used in genetics, hemostasis (e.g., factor V Leiden mutation detection), and infectious disease

K. DNA Sequencing

1. The order of nucleotides in DNA is determined.
2. Applications in the clinical laboratory include genotyping of microorganisms, detecting mutations, identifying human haplotypes, and determining polymorphisms.
3. Automated sequences have replaced manual methods.
4. **Pyrosequencing,** or sequencing by synthesis, is a commonly used method in automated DNA sequencers. In this method, a single strand of DNA to be sequenced is used as a template to enzymatically synthesize its complementary strand. Bases are added one at a time, and the instrument uses chemiluminescence to determine which base was actually added at each step.

V. CLINICAL LABORATORY APPLICATIONS

A. Analyte-Specific Reagents (ASRs)

1. ASRs are the raw materials (e.g., primers, probes, antibodies, and other test components) used in **"in-house"** diagnostic assays.
2. They are classified I, II, and III. Most molecular tests for infectious disease and tissue typing are **class I,** and their performance is established during test validation.
3. Can be used in FISH, PCR, HCA, and microarray analysis

B. Human Identity (DNA Polymorphisms)

1. **Methods:** RFLP, Southern blot, and PCR
2. **Targets:** STR, SNP, HLA, and mtDNA
3. **Applications:** Forensics and paternity, post–stem cell/bone marrow engraftment testing, linkage analysis of inherited (i.e., genetic) diseases, and tissue section identification

C. Detection, Identification, and Quantification of Microorganisms

1. **Methods:** HCA, NASBA, TMA, PCR, bDNA, RT-PCR, qPCR, SDA, and DNA sequencing
2. **Targets:** 16S and 23S rRNA, rDNA, housekeeping genes, toxin genes, antimicrobial resistance genes, interspersed repetitive elements, strain-specific sequences, and internal transcribed spacer elements
3. **Molecular epidemiology typing** during outbreaks, genotyping, and drug resistance screening
   a. Genotypic methods are highly reproducible and can discriminate between closely related organisms.
   b. Chromosomal RFLP analysis by pulsed field gel electrophoresis is commonly used for trace back studies during outbreaks of infectious diseases.
4. **Viral load (quantitative):** bDNA, NASBA, Amplicor RT-PCR, and qPCR; especially for human immunodeficiency virus, hepatitis C virus, hepatitis B virus, and cytomegalovirus
5. **Infectious disease testing of blood donor units:** Units of blood are screened for a number of bloodborne infectious agents using nucleic acid amplification tests.
6. **Bacteria frequently identified by molecular techniques**
   a. **Respiratory pathogens:** *Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Bordetella pertussis, Streptococcus pneumoniae,* and *Mycobacterium tuberculosis*
   b. **Urogenital pathogens:** *Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum, Mycoplasma genitalium, Mycoplasma hominis,* and *Haemophilus ducreyi.*

D. Molecular Detection of Inherited Diseases

1. **Chromosomal abnormalities:** Determine karyotype by FISH and comparative genomic hybridization
2. **Single gene disorders**
   a. **Methods:** DNA sequencing, PCR-RFLP, linkage analysis, Southern blot, LCR, and capillary electrophoresis
   b. **Factor V Leiden,** G to A change; resulting in substitution of the arginine (R) at position 506 by glutamine (Q), R506Q
      1) PCR-RFLP: Loss of *MnlI* site
      2) Single specific primer PCR (SSP-PCR): Sequence-specific primers

c. **Cystic fibrosis:** CFTR gene, chloride channel membrane protein
   1) **Most common change F508del,** about **1300 other mutations**
   2) xTAG® Cystic Fibrosis kit (Luminex Molecular Diagnostics) screens for 23 CFTR gene mutations and four polymorphisms by multiplex PCR and bead array.
   3) **Other methods:** RFLP, PCR-RFLP, heteroduplex analysis, temporal temperature gradient gel electrophoresis, SSP-PCR, bead array, and direct sequencing

3. **Trinucleotide repeat expansion disorders**
   a. Subset of STR with three bp repeating units that expand in length over generations.
   b. **Fragile X syndrome** is due to expanding copies of the CGG codon in the gene *FMR-1* located on the X chromosome. It results in mental retardation in males.
   c. **Huntington disease** is due to CAG expansion at 4p16.3.
4. **Molecular oncology**
   a. **Cell cycle:** G1 (cell growth), S (DNA synthesis), G2 (cell growth), and M (mitosis and cytokinesis)
   b. Gene and chromosomal mutations in solid tumors can be detected by SSP-PCR, single-strand conformation polymorphism analysis, direct sequencing, immunohistochemical staining, and FISH.
   c. **Translocation in hematologic malignancies**
      1) B cell leukemia and lymphomas, chronic myelogenous leukemia (CML), promyelocytic leukemia, and follicular lymphoma
      2) **Philadelphia (Ph) chromosome**
         a) Due to a translocation event: **t(9:22)(q34:q11)**
         b) Ph chromosome is associated with about 95% of the cases of CML. However, the presence of Ph is not specific for CML. It is also found in acute lymphoblastic leukemia (25–30% in adult and 2–10% in pediatric cases) and occasionally in acute myelogenous leukemia.
         c) **Detection methods:** Karyotype for Ph chromosome by FISH, Southern blot, capillary gel electrophoresis, and real-time PCR for quantitative estimation of treatment response

review questions

INSTRUCTIONS Each of the questions or incomplete statements that follows is comprised of four suggested responses. Select the *best* answer or completion statement in each case.

1. If 20% of the nucleotides in an organism are adenine, predict the percentage of nucleotides that are guanine.
   A. 20%
   B. 30%
   C. 40%
   D. 60%

2. Which of the following is *not* required for DNA replication by PCR?
   A. Oligonucleotide primers
   B. DNA polymerase
   C. DNA ligase
   D. Deoxynucleotides

3. In naming restriction endonucleases, the first letter of the name comes from the
   A. Bacterial genus
   B. Bacterial species
   C. Scientist who discovered it
   D. Geographic location of its discovery

4. A restriction enzyme recognizes the sequence, 5' CT^ATAG 3', and cuts as indicated. Predict the ends that would result on the *complementary* DNA strand.
   A. 3' G 5' 3' ATATC 5'
   B. 3' GA 5' 3' TATC 5'
   C. 3' GATA 5' 3' TC 5'
   D. 3' GATAT 5' 3' C 5'

5. The absorbance of a 1:100 dilution of isolated dsDNA solution, measured at 260 nm, is 0.062. What is a reasonable estimate for the dsDNA concentration of the sample, expressed in μg/mL?
   A. 3.1
   B. 6.2
   C. 310
   D. 5000

6. In the isolation of RNA, diethylpyrocarbonate (DEPC) is used to
   A. Inhibit RNase
   B. Lyse the cells
   C. Precipitate the DNA
   D. Remove buffer salts

7. Purification resins used to isolate DNA take advantage of the fact that DNA is
   A. Double stranded
   B. Negatively charged
   C. Higher in concentration than RNA
   D. Higher molecular weight than RNA

8. After performance of DNA electrophoresis, the isolated bands in the kilobase size range appear too close together. Which of the following can be done with the next run to improve the appearance/separation of the bands in the samples?
   A. Increase the percent agarose concentration of the matrix
   B. Increase the running time of the electrophoresis assay
   C. Increase the sample volume applied to the gel
   D. Decrease the sample volume applied to the gel

9. Which of the following is commonly used as a label in molecular tests?
   A. Biotin
   B. DNase
   C. RNase
   D. ^{125}I

10. Which of the following is *not* an example of target amplification?
    A. Reverse transcription-PCR (RT-PCR)
    B. Transcription mediated amplification (TMA)
    C. Branched chain DNA amplification (bDNA)
    D. Polymerase chain reaction (PCR)

11. In forensic testing, DNA fingerprinting can identify individuals with high accuracy because
    A. Human genes are highly conserved
    B. Only a small amount of sample is needed
    C. Human gene loci are polymorphic
    D. DNA is stable and not easily contaminated

12. The technique that makes ssDNA from an RNA template is called
    A. Strand displacement amplification
    B. Polymerase chain reaction
    C. Ligase chain reaction
    D. Reverse transcription

13. A 5850-base plasmid possesses *Eco*RI restriction enzyme cleavage sites at the following base pair locations: 36, 1652, and 2702. Following plasmid digestion, the sample is electrophoresed in a 2% agarose gel. A DNA ladder marker, labeled M in Color Plate 56■, is included in the first lane, with base pair sizes indicated in lanes A through D. Which lane represents the sample pattern that is most likely the digested plasmid?
    A. A
    B. B
    C. C
    D. D

14. Which of the following is characteristic of DNA chips (i.e., DNA microarrays)?
    A. Allow detection and discrimination of multiple genetic sequences at the same time.
    B. Thousands of oligonucleotide probes are labeled and placed on glass or silicon surfaces.
    C. Unlabeled target sequences within the patient sample are detected by hybridization to labeled probes.
    D. All the above

15. The most useful feature of the molecules streptavidin and biotin is that they bind
    A. Specifically to nucleic acids
    B. Only in neutral pH conditions
    C. To each other with very high affinity
    D. Directly to DNA immobilized on nitrocellulose

16. What is the theoretic estimation of the number of DNA target sequences present (per original double-stranded DNA in solution) following 15 cycles of PCR?
    A. 30
    B. 2^{10} (i.e., 1024)
    C. 2^{15} (i.e., 32,768)
    D. 2^{20} (i.e., 1,048,576)

17. "Star activity" for a restriction enzyme refers to
    A. An ability to cleave DNA at sequences different from their defined recognition sites
    B. The enzyme's specificity for sites of methylation within the nucleotide sequence
    C. The temperature and pH conditions at which the enzyme will function optimally
    D. The percent increased accuracy of the enzyme when placed in ideal conditions of pH

18. What enzyme recognizes and cuts overlapping DNA sequences formed between mutant or normal probes and target sequences within samples?
    A. Restriction endonuclease
    B. DNA ligase
    C. Cleavase
    D. RNase H

19. If a DNA probe is added to nitrocellulose after the transfer step but before the blocking step, which of the following will occur?
    A. The probe will nonspecifically bind to its DNA target.
    B. Unoccupied spaces on the nitrocellulose will bind the probe.
    C. The DNA target on the nitrocellulose will be unable to bind the probe.
    D. Bound probe will be washed away in the next wash step.

20. Which of the following items is *not* used in the preparation of a DNA probe for Southern blotting using random hexamer primers?
    A. Template DNA
    B. Three unlabeled deoxynucleotides
    C. Dideoxynucleotides, with one of them labeled
    D. DNA polymerase

21. Which of the following is considered a "high stringency" condition for DNA probe protocols?
    A. Using wash buffer with highly acidic pH
    B. Washing the matrix with high-salt buffer
    C. Radiolabeling the probe with ^{35}S rather than ^{32}P
    D. Washing the transfer membrane (e.g., nitrocellulose or nylon) at high temperature

22. When compared to Southern blot hybridization testing, PCR
    A. Is less sensitive to DNA degradation than Southern blot
    B. Includes transfer of DNA onto a nylon membrane
    C. Requires no specialized equipment
    D. Is more labor intensive

23. Which of the following specimen types is *not* used routinely as source material for molecular genetic tests?
    A. Whole blood
    B. Buccal scrapings
    C. Amniocytes
    D. Rectal swabs

24. In the presence of salt, DNA is precipitated from solution by
    A. 10 mM Tris, 1 mM EDTA
    B. 0.1% sodium dodecyl sulfate (SDS)
    C. Alkaline buffers, such as 0.2 N NaOH
    D. Alcohols, such as 95% ethanol or isopropanol

25. TaqMan probes used to increase specificity of real-time PCR assays generate a fluorescent signal
    A. At the beginning of each cycle during the denaturation step
    B. When the probes bind to the template (i.e., during annealing)
    C. When the probe is digested by 5' → 3' exonuclease activity during extension of primers (i.e., DNA synthesis)
    D. When the reporter fluorophor on the probe is separated from the quencher molecule by a restriction enzyme

26. For the purpose of diagnosing genetic diseases, what component of whole blood is used for the extraction of DNA?
    A. Leukocytes
    B. Plasma
    C. Platelets
    D. Red blood cells

27. Which of the following statements best describes characteristics of RNase?
    A. It degrades mRNA but not rRNA.
    B. It is found in large concentrations on hands.
    C. Its activity can be eliminated by autoclaving.
    D. Its activity occurs in a limited temperature range between 25 and 65°C.

28. Which of the following is the *least* likely inhibitor of PCR?
    A. Heme
    B. Sodium heparin
    C. DEPC (diethylpyrocarbonate)
    D. EDTA (ethylenediaminetetraacetic acid)

29. Frequently, DNA probes are used to detect target sequences in Northern or Southern blots. Hybridization occurs between DNA probe and RNA or DNA on the blot, respectively. To ensure that only exactly matched complementary sequences have bound together, the blot is washed under stringent conditions. Stringency of the wash steps to remove unbound and mismatched probe can be increased by
    A. High temperature, high NaCl concentration, and high detergent (i.e., SDS) solution
    B. High temperature, low NaCl concentration, and high detergent (i.e., SDS) solution
    C. High temperature, high NaCl concentration, and low detergent (i.e., SDS) solution
    D. Low temperature, high NaCl concentration, and high detergent (i.e., SDS) solution

30. In RNA, which nucleotide base replaces thymine of DNA?
    A. Adenine
    B. Cytosine
    C. Guanine
    D. Uracil

31. The component parts of a dNTP include a purine or pyrimidine base, a
    A. Ribose sugar, and one phosphate group
    B. Deoxyribose sugar, and three phosphate groups
    C. Ribose sugar, and two phosphate groups
    D. Deoxyribose sugar, and two phosphate groups

32. When comparing two dsDNA sequences of equal length, the strand that has a higher
    A. G + C content has a higher melting temperature (T_m)
    B. A + T content has a higher T_m
    C. A + T content has more purines than pyrimidines along its length
    D. G + C content has more purines than pyrimidines along its length

33. Molecular typing of bacterial strains is based on restriction fragment length polymorphisms (RFLPs) produced by digesting bacterial chromosomal DNA with restriction endonucleases. Which of the following techniques is used to separate the large DNA fragments generated?
    A. Ribotyping
    B. DNA sequencing
    C. Pulsed field gel electrophoresis
    D. Reverse transcription–polymerase chain reaction

34. Which of the following amplification methods does *not* employ isothermal conditions?
    A. Nucleic acid sequence–based amplification (NASBA)
    B. Polymerase chain reaction (PCR)
    C. Strand displacement amplification (SDA)
    D. Transcription mediated amplification (TMA)

35. The coding region of a human gene is called
    A. Exon
    B. Intron
    C. SNP
    D. VNTR

36. The central dogma is that DNA is used to make RNA, which is then used to make protein. In this scheme the two processes that are involved (i.e., DNA to RNA and RNA to protein) are termed
    A. Replication and transcription
    B. Synthesis and encryption
    C. Transcription and translation
    D. Initiation and elongation

37. How many chromosomes are contained in a normal human somatic cell?
    A. 22
    B. 23
    C. 44
    D. 46

38. An ordered sequence of events makes up the cell cycle. Which of the following describes the correct sequence of events starting at G1?
    A. G1, G2, S, M
    B. G1, S, G2, M
    C. G1, M, G2, S
    D. G1, S, M, G2

39. Purified DNA remains stable indefinitely when stored as
    A. Small aliquots at 4°C
    B. Large aliquots at 25°C
    C. Small aliquots at –70°C
    D. Large aliquots at –20°C

40. In Color Plate 57■ the procedure of Southern blotting is diagrammed. In the upper panel, restricted genomic DNA fragments have been separated by electrophoresis in an agarose gel. In lane 1 is a molecular weight marker, in lanes 2–4 are three patient samples, and in lane 5 is a positive control DNA sequence for the probe used. After electrophoresis, DNA was transferred from the gel onto a nylon membrane and then hybridized with a radiolabeled probe that recognizes CGG trinucleotide repeat. Fragile X syndrome is the most frequently inherited form of mental retardation in males (1:1000–1:1500 individuals). In affected individuals, expansions of the trinucleotide repeat within the fragile X gene increase to greater than 200 repeats. The bottom panel shows the resultant autoradiogram after a series of high-stringency washes. The three patient samples (lanes 2–4) are DNA from individuals of a single family, one of them suffering from fragile X syndrome. In which lane is the mentally handicapped patient's sample?
    A. Lane 2
    B. Lane 3
    C. Lane 4
    D. Cannot be determined by the results given

41. An advantage of amplification technologies for clinical laboratories is that
    A. They require inexpensive test reagents
    B. They lend themselves to automated methods
    C. Each target molecule sought requires a unique set of primers
    D. Contamination is not a concern when performing these assays

42. The assay method that detects the expression of a gene rather than the mere presence or structure of a gene is termed
    A. RT-PCR
    B. TMA
    C. Multiplex PCR
    D. Ribotyping

43. Which of the following assays *cannot* be accomplished using PCR methods employing only *Taq* polymerase?
    A. Diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection
    B. Detection of single base pair gene mutations, such as in cystic fibrosis
    C. Detection of HLA-A, B and DR genotypes
    D. Determination of viral load for HCV

44. One method to prevent "false-positive" PCR results includes the use of dUTP in the reaction mix, resulting in amplicons containing U in place of T. The enzyme used to decrease contamination is
    A. Uracil-*N*-glycosylase
    B. *Taq* polymerase
    C. S1 nuclease
    D. DNase

For questions 45–47, refer to Color Plates 58a■ and b■.

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45. What temperature is best for use in Step 1?
    A. 35°C
    B. 55°C
    C. 75°C
    D. 95°C

46. What temperature range is most appropriate for Step 2?
    A. 25–35°C
    B. 55–65°C
    C. 70–80°C
    C. 90–100°C

47. What substance within the PCR mix influences the accuracy of cDNA?
    A. Oligonucleotide primers
    B. Monovalent cation K^+
    C. Divalent cation Mg^{2+}
    D. Deoxyribonucleotide triphosphate molecules

48. The following question refers to Color Plate 59■. Factor V Leiden mutation causes increased risk of thrombosis. It is caused by a single base mutation in which guanine (G) is substituted for adenine (A) with a subsequent loss of a restriction site for the enzyme *Mnl*I. Primers used in this example generate a 223 bp PCR product from patient DNA. After resulting PCR products are digested with *Mnl*I, normal patients produce the following DNA fragments: 104 bp, 82 bp, 37 bp. In Color Plate 59■, the 37 bp fragment is not seen in all lanes because it is sometimes below detectable levels. Lane identities are as follows: M (molecular weight marker), 1–5 (patient 1 to patient 5, respectively), + (positive control showing 104, 82, and 37 bp fragments), Neg (sterile water used in place of sample DNA). Which patient is heterozygous for the factor V Leiden mutation?
    A. Patient 1
    B. Patient 2
    C. Patient 3
    D. Patient 4

49. The translocation resulting in the Philadelphia chromosome is detected by
    A. Southern blot analysis only
    B. Cytogenetic analysis (e.g., karyotyping) only
    C. PCR, Southern blot, and cytogenetic analysis
    D. RT-PCR, Southern blot, and cytogenetic analysis

50. Which sample in Color Plate 60■ contains the largest amount of cytomegalovirus?
    A. Sample 4
    B. Sample 5
    C. Sample 11
    D. Only qualitative results can be determined in this assay.

answers & rationales

1.

B. Because of the base pairing property within DNA, the presence of 20% adenine (A) means there must also be 20% thymine (T) in the organism. This means 40% of the DNA is A or T, leaving 60% of the DNA to be cytosine (C) or guanine (G). Because there must be an equal amount of each base type within the base pair, 60% divided by 2 gives 30% each of cytosine and guanine.

2.

C. DNA ligase is an enzyme that catalyzes the reaction between the 5'-phosphate end of one DNA fragment and the 3'-hydroxyl end of the next. This "nick sealing" requires energy from ATP hydrolysis, thus remaking the broken phosphodiester bond between the adjacent nucleotides. Ligase is a very important enzyme in DNA repair, but it is not used in a polymerase chain reaction (PCR). PCR does require a DNA template, two primers to anneal to nucleotide sequences flanking the desired amplification sequence, deoxynucleotide triphosphates (dNTPs) to be used as building blocks for the growing DNA chain, DNA polymerase, and magnesium chloride as an essential cofactor for DNA polymerase activity.

3.

A. The first letter of a restriction endonuclease's name comes from the bacterial genus from which it originated. The second and third letters derive from the bacterial species. The last letter indicates the subspecies or strain from which the enzyme was obtained. The last Roman numeral represents the numerical place the enzyme has among those which have been isolated from that bacterial genus/species/strain. For example, *Eco*RI is the first restriction endonuclease isolated from the bacterium *Escherichia coli,* strain R, whereas *Eco*RV is the fifth such enzyme to be discovered.

4.

C. The complementary strand for this DNA sequence would be, read left to right, 3' GATATC 5'. Restriction endonucleases require dsDNA, because they use as their substrate palindromic molecules, meaning a molecule that will "read" the same left to right or right to left. In this instance, the complementary strand, read 5' to 3' (right to left), reads the same as the sense strand, read 5' to 3'. If the enzyme cuts the sense strand as indicated, between the thymine and adenine, it will cut the complementary strand identically. This will leave, on the sense strand, the two sequences 5' CT 3' and 5' ATAG 3'. The complementary strand will show 3' GATA 5' and 3' TC 5'. Check again to see that these new sequences actually are identical, read 5' to 3', on both strands.

5.

C. The concentration of dsDNA can be estimated by taking its absorbance reading at 260 nm and multiplying that absorbance by a factor of 50, because one absorbance unit at 260 nm equals approximately 50 μg/mL. To solve this problem: 100 (dilution factor) × 0.062 (sample Abs. at 260 nm) × 50 μg/mL (conversion factor for dsDNA) = 310 μg/mL.

6.

A. In the isolation of RNA, it is very important to remove all RNase activity. Such enzymes are considered ubiquitous, so precautions must always be taken. Diethylpyrocarbonate (DEPC), or diethyl oxydiformate, will inactivate RNase, thus protecting RNA from degradation. It is used in solution at 0.1–0.2% (w/v) concentration.

7.

B. Resin-based purification of DNA takes advantage of the fact that DNA, at alkaline pH, possesses a net negative charge. Cells are incubated with detergent, which causes their lysis. RNA is digested with RNase, and the solution is neutralized with potassium acetate. This salt solution will precipitate the detergent and all proteins. Lysate is added to the exchange resin, and both DNA and residual RNA will bind. RNA and ssDNA are removed with a wash buffer, and the desired dsDNA will be eluted from the resin with either water or pH 8.0–8.5 buffer.

8.

B. The rate of electrophoretic separation when using polyacrylamide or agarose gels is affected by time, current, and the percent matrix used. Sample volume will not affect rate of separation but only makes the resulting bands more visible when stained. Achieving increased separation can be accomplished by increasing the time or current used. It can also be achieved by decreasing the percent matrix, because the "pores" present in a 1% agarose gel will be larger than those in a 5% gel. This larger size pore will allow easier molecular passage of DNA molecules during electrophoresis. Conversely, achieving a tighter band pattern (i.e., higher resolution of smaller DNA molecules) can be accomplished by decreasing time or current, or increasing percent matrix used.

9.

A. There are three essential parts to any molecular test performed: (1) a target, (2) a probe, and (3) a signal that can be detected. There are many ways a probe can be labeled in order for a signal to be produced and an analyte measured. Radioactive isotopes, such as ^{32}P, ^{33}P, ^{35}S, and ^{125}I, have traditionally been used to label probes. Positive signals are measured using X-ray exposure or scintillation counting. However, because of environmental factors, costs, and safety concerns, radioactive labels are being used with decreasing frequency. Nonradioactive probes are often labeled with haptens (e.g., digoxigenin), biotin, fluorescein, rhodamine, or a chemical such as acridinium esters. Detection of the hybridization (i.e., a positive test) is dependent on the type of label used, but it is generally colorimetric, fluorescent, or chemiluminescent. Hapten-labeled and biotin-labeled probes are detected by enzyme-conjugated antihapten antibodies and enzyme-conjugated streptavidin. Enzyme conjugates used are horseradish peroxidase and alkaline phosphatase.

10.

C. Target amplification refers to a process that increases the number of copies of the target DNA or RNA nucleotide sequence. Examples include the polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), transcription-mediated amplification (TMA), and nucleic acid sequence–based amplification (NASBA). Signal amplification will cause increased signal strength without increasing the number of target molecules. One example of signal amplification is the branched chain DNA reaction. Probe amplification will increase the number of copies of the probe that is complementary to the target. One example of probe amplification is the ligase chain reaction.

11.

C. Although human genes are highly conserved in gene coding regions, human gene loci are polymorphic, which means many forms of the gene can exist at a given locus, making each person "unique." Only identical twins are not "unique." Short tandem repeats (STRs) account for the many polymorphisms used in DNA fingerprinting. STRs are short, repetitive sequences of 3–7 base pairs and are abundant in the human genome. There are STR kits commercially available from several manufacturers. The common loci used in forensics to obtain DNA fingerprints are the CTT triplex and the FFv triplex. These contain the following loci: CSF1PO (proto-oncogene *CSF-1*), *TPOX* (thyroid peroxidase gene), *TH01* (tyrosine hydroxylase gene), *F13A01* (coagulation factor XIII gene), *FES/FPS* (proto-oncogene *c-fes/fps*), and *v-WA* (von Willebrand gene). This testing does not require large quantities or high-quality DNA for successful results. It uses PCR, which is highly sensitive; however, this characteristic also makes the PCR method prone to contamination.

12.

D. The process whereby a strand of RNA is synthesized from template DNA is called transcription. The enzyme involved is RNA polymerase. It is possible, however, as retroviruses have shown, to produce DNA using template RNA. This reversal of the central nucleic acid dogma is called "reverse transcription," and the enzyme that performs this is called reverse transcriptase. After synthesizing a single-stranded DNA molecule from RNA, a different enzyme (DNA polymerase) then synthesizes a complementary strand to produce a DNA double helix. The other three answers describe amplification methods designed to increase the sensitivity of molecular diagnostic tests. They accomplish this by making copies of either the target nucleic acids (e.g., PCR), or the probe molecules (e.g., ligase chain reaction and strand displacement amplification), or the signal produced (e.g., branched chain DNA reaction).

13.

C. To solve this problem, it is necessary to recognize that plasmid DNA exists as a closed circle. This means that base pair #1 is adjacent to base pair #5850. If the enzyme cleaves the plasmid at positions 36, 1652, and 2702, this will result in three pieces of DNA. One piece will contain base pairs (bp) 37 through 1652 (with a size of 1616 bp), a second will contain bp 1653 through 2702 (with a size of 1050 bp), and the third will span the sequence from bp 2703 through 5850 and from 1 to 36 (with a size of 3184 bp). Note that to determine the size of each piece, subtract the numbers corresponding to each adjacent cut site (e.g., 1652 − 36 = 1616 and 2702 − 1652 = 1050). For the third piece, subtract the highest numbered cut site (i.e., 2702) from the total size of the plasmid (i.e., 5850), and add the size of the piece beginning at bp #1 through bp #36. Use the DNA ladder marker (lane M) in Color Plate 56■ to predict the placement of these pieces (3184 bp, 1616 bp, and 1050 bp) of DNA on the final electrophoresis pattern.

14.

A. DNA chips (i.e., DNA microarrays) allow detection and discrimination of multiple genetic sequences at the same time. DNA chips have thousands of oligonucleotide probes arranged on glass or silicon surfaces in an ordered manner. Target sequences within the patient sample are fluorescently labeled in solution. The labeled sequences in solution are then incubated with the DNA chips containing the oligonucleotide probes attached to the silicon or glass surface. Hybridization will occur between labeled complementary sequences within the patient samples and their corresponding probe on the chip. The DNA chip is placed in an instrument that scans the surface with a laser beam. The intensity of the signal and its location are analyzed by computer and provide a quantitative description of the genes present. Because placement of the oligonucleotides is known, identification of the gene or organism may be determined.

15.

C. Biotin is a vitamin involved physiologically in single carbon transfers. Streptavidin is a protein derived from *Streptomyces avidinii,* consisting of four subunits, each of which can bind one biotin molecule. This bond formation is rapid and essentially irreversible. The interaction between streptavidin and biotin is the strongest known noncovalent biologic interaction between a protein and its ligand. *In vitro* assays take advantage of this strong and specific binding by covalently attaching streptavidin to a reporter molecule (e.g., a primary antibody) and then incubating this with a secondary fluorescent-labeled antibody conjugated to biotin. Each streptavidin molecule will bind four biotin-conjugated molecules, thereby increasing fourfold the signal generated.

16.

C. Assuming 100% efficiency, each cycle of the polymerase chain reaction doubles the number of DNA molecules present in the solution. Starting with one DNA template molecule, there would be $2^2 = 4$ DNA molecules present after two cycles. After 5 cycles, this would result in $2^5 = 32$. Based on a starting single molecule of double-stranded DNA, after 15 cycles there would theoretically be 2^{15} molecules (32,768). Actual yield is somewhat less than theoretical yield because PCR products created in the first two PCR cycles are slightly longer than the target amplicon. Thus, yield may be better calculated as $2^{(n-2)}$. Actual yield may be decreased by a plateau effect that may occur in later PCR cycles when some components of the PCR become reaction limiting.

17.

A. Restriction enzymes will show specificity for a target nucleotide sequence when used under optimal conditions of temperature and glycerol, salt, and substrate concentrations. If these conditions are not optimal, some enzymes will lose their specificity and begin to cleave more randomly. This undesirable, nonoptimal digestion is called "star activity." Such activity is evident when the following parameters are altered in the reaction environment: more than 100 units of enzyme per microgram of DNA, more than 5% glycerol content, less than 25 mM salt concentrations, pH >8.0, presence of dimethyl sulfoxide (DMSO), ethanol, or other organic solvents.

18.

C. Cleavase is an enzyme isolated from bacteria that is likely important in DNA repair *in vivo*. The enzyme recognizes overlapping sequences of DNA and cleaves in the overlapping sequence. Third Wave Technologies has exploited the use of this enzyme in their Invader® system. Target nucleic acid is mixed with Invader and signal probes. When the Invader and signal probes bind the target, the 5' end of the signal overlaps with the Invader probe, and cleavase cleaves the signal probe. In the next step, the cleaved signal probe binds a fluorescent-labeled reporter probe containing complementary sequences and a quencher molecule, thus forming an overlapping structure. This molecule is subsequently cut by cleavase, which removes the reporter molecule from the quencher. The signal generated is directly related to the amount of target sequences in the original sample. Restriction endonucleases are also bacterial enzymes that recognize specific sequences within DNA and cut DNA near or within the recognized sequence. DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 3' hydroxyl and 5' phosphate groups of adjacent nucleotides. RNaseH hydrolyzes RNA strands of a RNA:DNA hybrid molecule.

19.

B. After DNA is transferred to a nitrocellulose or nylon membrane, many sites on the membrane will not be occupied. Adding a probe at this point will not only allow for specific binding of the probe to the target DNA sequence, but also the nonspecific binding of the probe to the available binding sites on the membrane. This will cause nonspecific signal generation throughout the matrix. To prevent this, the membrane must first be treated with blocking agents. Denhardt solution and denatured nonhomologous DNA (e.g., salmon sperm DNA) are often used to bind up all the available sites on the matrix and allow for specific binding of the probe in the next step.

20.

C. Preparation of a DNA probe using random hexamer primers requires a DNA template containing the desired target sequence; four deoxynucleotides, at least one of which must be labeled (e.g., radionuclide, fluorescent, biotin, etc.); and DNA polymerase. The double-stranded DNA template (25–50 ng) is denatured, and a mixture of random oligonucleotides of six bases in length anneal to the template DNA. The primers are extended by the action of a DNA polymerase (e.g., T7 DNA polymerase) in the presence of one or two labeled deoxynucleotide triphosphates. Random primed probes are generally 500 nucleotides in length. Solutions containing the labeled probes are incubated with the blot. Hybridization of the labeled probe will occur if the gene being sought is present in the DNA on the blot to give a positive signal. Dideoxynucleotides are used in preparing samples for DNA sequencing by the Sanger method and cause DNA polymerization to cease.

21.

D. In Southern blots, hybrids can form between molecules with similar but not necessarily identical sequences. The washing conditions used after adding the labeled probe can be varied so that hybrids with differing mismatch frequencies are controlled. The higher the wash temperature or the lower the salt concentration in the wash buffer, the higher the stringency. Increasing the stringency will decrease the number of mismatches that form between the probe and the target DNA.

22.

A. Standard Southern blot techniques recommend the use of 10 μg of high-quality genomic DNA when studying single-copy genes. In a subsequent step the genomic DNA is restricted (i.e., cut into small fragments of predictable size). The resulting fragments of the gene of interest generally range in size from 1.0 to 10.0 kilobases. In contrast, the gene sequence of interest to be amplified in a routine PCR targets a smaller portion of the gene (generally 150–500 bases in length). Because the target is a smaller size, partially degraded DNA (i.e., genomic DNA samples of lesser quality) can be amplified successfully. Long-range PCR methods are available that extend the range of PCR products synthesized from 5 to 35 kilobases. Because PCR targets are usually a few hundred bases in length, high-molecular-weight DNA is not necessary for successful PCR. It requires a thermocycler to take the reaction through the cycles of three temperatures needed for denaturation, hybridization, and elongation steps. Turn-around time is also an advantage of PCR reactions because results can be completed in less than 4 hours, whereas Southern blotting takes up to 1 week to complete because of multiple steps required for this procedure.

23.

D. Many frequently used protocols in molecular biology involve PCR. Several substances can inhibit this reaction. For example, because of the nature of fecal material, it is not routinely used, and materials in swabs have also been reported to inhibit PCR. Therefore, a more appropriate specimen that could be used for PCR would be a stool filtrate. Nucleated cells are necessary for isolation of DNA. Whole blood is an acceptable specimen. White blood cells are the source of DNA in this type of specimen and must be separated from red blood cells as soon as possible because hemoglobin will inhibit PCR. For diagnosis of blood parasites, such as *Babesia* and *Plasmodium,* a hemolyzed and washed red blood cell sample is preferred for recovery of the DNA from the parasites. Amniocytes are used for molecular cytogenetic testing to prenatally screen for genetic diseases. Noninvasive collection of cells for genetic and forensic testing can be obtained from the buccal (oral) mucosa.

24.

D. Alcohol precipitation of nucleic acids is a standard method in molecular biology. Sterile water, 10 mM Tris, 1 mM EDTA, or 0.1% SDS can be used to rehydrate DNA; 1 mM EDTA and 0.1% SDS are included in these mixes to inhibit DNases. Alkaline solutions, such as 0.2 N NaOH, are used to denature nucleic acids.

25.

C. Real-time PCR or quantitative PCR (qPCR) is a modification of PCR that allows quantification of input target sequences without addition of competitor templates or multiple internal controls. qPCR is used to measure copy numbers of diseased human genes and viral and tumor load and to monitor treatment effectiveness. The accumulation of double-stranded PCR products during PCR as they are generated can be measured by adding fluorescent dyes that are dsDNA-specific to the reaction mix, such as SYBR green. However, misprimed products or primer dimers will also generate fluorescence and give falsely high readings. Thus, more specific systems utilizing probes to generate signal, such as the TaqMan probes, Molecular Beacons, and Scorpion-type primers, have been developed. In the TaqMan probe-based system, specific primers are present to prime the DNA synthesis reaction catalyzed by *Taq* polymerase, thus forming the cDNA product. The TaqMan probe binds to a smaller region within the target sequence. The TaqMan probe has a 5' reporter fluorophor and 3' quencher molecule. During extension of the primers by *Taq* polymerase to form cDNA product (i.e., DNA synthesis), the 5' → 3' exonuclease activity of *Taq* polymerase digests the TaqMan probe separating the reporter molecule from the quencher to generate a fluorescent signal. Molecular Beacon probes form hairpin structures due to short inverted repeat sequences at each end. The probe has a reporter dye at its 5' end and a quencher dye at its 3' end. In the unbound state, fluorescence is suppressed because reporter and quencher dyes are bound closely together by the short inverted repeat sequences. In qPCR assays, fluorescence occurs when molecular beacon probe binds the denatured template during the annealing step because reporter dye is separated from the quencher molecule. Scorpion primers, which contain a fluorophore and a quencher, are covalently linked to the probe. In the absence of the target, the quencher absorbs fluorescence emitted by the fluorophore. During the PCR reaction, in the presence of the target, the fluorophore and the quencher of the Scorpion primers separate, resulting in an increase in the fluorescence emitted. All of these systems require excess concentrations of the labeled probe/primer, so fluorescence emitted is directly proportional to the amount of template available for binding.

26.

A. Leukocytes are routinely used for extraction of DNA from human blood. Mature red blood cells and platelets have no nuclei. Plasma or serum can be used for detection of viremia, but it is not used for analysis of genetic diseases.

27.

B. The highest concentration of RNase is found on hands; thus, it is imperative that gloves be worn when working with RNA. RNases are ubiquitous and can act at temperatures below freezing (–20°C) and above boiling. For long-term storage, purified RNA is best stored at −70°C or below. RNases plague experiments in which RNA is used. Simple autoclaving does not eliminate RNase activity. To remove RNases, glassware must be pretreated with an RNase inhibitor, such as DEPC, followed by autoclaving; alternatively, baking glassware in a >250°C oven for 4 hours will destroy RNase. To prevent RNA degradation, isolation of RNA should be done using chaotropic agents (e.g., guanidine isothiocyanate) that inhibit RNase activity. When analyzing RNA in a gel, formaldehyde or other agents that denature RNases must be included in the gel. High-quality (i.e., undegraded) RNA will appear as a long smear with two or three distinct areas that correspond to the ribosomal RNA subunits: 28S (~4800 bases), 18S (~1800 bases), and 5.8S (~160 bases), whereas degraded RNA will appear as a smear at the bottom of the gel.

28.

D. EDTA and ACD (acid citrate dextrose) are the preferred anticoagulants for specimens that will undergo PCR. These reactions can be inhibited by a variety of substances. PCR inhibitors are concentration dependent; inhibition can often be overcome by simply diluting the DNA sample. Heme and sodium heparin can inhibit PCR. However, laboratory methods can be used to remove these inhibitors, if necessary. Diethylpyrocarbonate (DEPC) is a substance used to inhibit RNases; it can also inhibit PCR.

29.

B. Stringency of hybridization is accomplished at two steps in the blotting technique. The first step is hybridization conditions of the labeled probe in solution with the transferred RNA or DNA targets on the membrane. The second step occurs when the membrane is washed to remove unbound probe. In the hybridization reaction, formamide and temperature can be used to increase stringency. During wash steps, increasing temperature and increasing detergent concentration (e.g., 1% SDS) will increase stringency; whereas lowering NaCl concentration also increases stringency. At the end of the highest stringency wash, only specific hybrids of interest should remain on the blot.

30.

D. The four nucleotide bases found in RNA are adenine (A), guanine (G), cytosine (C), and uracil (U). The purines A and G are the same as in DNA. C is present in both DNA and RNA; however, in RNA, the DNA nucleotide base thymine (T) is replaced by uracil (U). RNA is usually single stranded, although double-stranded areas can occur. A pairs with U, and C pairs with G.

31.

B. dNTP stands for deoxyribonucleotide triphosphate. Nucleotides are the building blocks of nucleic acids. They are composed of phosphate groups, a 5-sided sugar molecule, and a nitrogenous base. Nitrogenous bases are either purines (A, G) or pyrimidines (C, T, or U, an RNA-specific base). The sugar molecules are either ribose (in RNA) or deoxyribose (in DNA), with the only difference in structure being the lack of a hydroxyl group at position 2' in the deoxyribose molecule. When the sugar is bound to a base without the phosphate group, the molecule is called a nucleoside. A nucleotide can have 1, 2, or 3 phosphate groups, which are termed monophosphate, diphosphate, and triphosphate, respectively.

32.

A. DNA is composed of two strands of polynucleotides coiled in a double helix. The outside backbone is composed of sugar-phosphate moieties, whereas the purine and pyrimidine bases are stacked inside the helix. The size and stability of the DNA molecule is such that only specific bases can hydrogen bond to each other to hold the two strands together (A-T, C-G, and vice versa). This is referred to as complementary base pairing. An A-T base pair is less stable than a C-G base pair, because three hydrogen bonds form between C-G and only two hydrogen bonds form between A-T. The increased stability between C-G causes the melting temperature (T_m) to be greater in a double-stranded DNA (ds DNA) segment with more C-G pairs than a segment with more A-T pairs. In all dsDNA molecules, the number of purines (A + G) equals the number of pyrimidines (C + T).

33.

C. Pulsed field gel electrophoresis (PFGE) is used to separate extremely large DNA molecules by placing them in an electric field that is charged periodically in alternating directions, forcing the molecule to reorient before moving through the gel. Larger molecules take more time to reorient; thus they move more slowly. Bacterial DNA is digested by restriction enzymes in agarose plugs. The PFGE of the digested fragments provides a distinctive pattern of 5 to 20 bands ranging from 10 to 800 kilobases. DNA sequencing determines the exact nucleotide sequence base by base of any organism; however, it is too laborious for epidemiologic purposes. Ribotyping is a Southern blot type of analysis using rRNA probes to detect ribosomal operons (i.e., sequences coding for 16S rRNA, 25S rRNA, and one or more tRNAs) of individual bacterial species. Its discriminatory power is less than PFGE. Reverse transcription–polymerase chain reaction (RT-PCR) is a method that determines whether a gene is being expressed. The starting material for RT-PCR is ssRNA.

34.

B. PCR requires a thermocycler because cycling at three different temperatures is the basis for this technique. First, template DNA (i.e., which may contain the target sequence) is denatured at 94°C. Next, the temperature is lowered to allow specific primers to anneal to the single-stranded target, generally at temperatures near 55°C. In the third portion of the cycle, primers are extended using deoxynucleotide triphosphate molecules to form a complementary copy of DNA under the direction of a thermostable DNA polymerase enzyme, such as *Taq* polymerase. The optimal temperature at which *Taq* polymerase acts to extend the primers is 72°C. Thus, at the end of one cycle, one molecule of dsDNA has now become two molecules of dsDNA. Cycles are generally repeated about 30 times to theoretically yield 2^{30} DNA molecules. The three steps of each cycle are termed denaturation (94°C), annealing of primers (~55°C), and extension of primers (72°C). The other methods listed, nucleic acid sequence–based amplification, strand displacement amplification, and transcription mediated amplification, are also amplification methods; however, they have been modified so all reactions take place at a single temperature (isothermal).

35.

A. The coding regions of eucaryote genes are called exons. The noncoding intervening regions are called introns. In eucaryotes, the introns and exons are transcribed into mRNA; however, before mRNA is translated, the introns are removed and the exons are spliced together. SNP is an abbreviation for single nucleotide polymorphism, and VNTR refers to variable number tandem repeats.

36.

C. Central dogma describes the flow of genetic information from DNA to RNA to protein. Individual DNA molecules serve as templates for either complementary DNA strands during replication or complementary RNA molecules during transcription. In turn, RNA molecules serve as templates for ordering of amino acids by ribosomes to form polypeptides during protein synthesis, also known as translation.

37.

D. DNA in human somatic cells is compartmentalized into 22 pairs of chromosomes, referred to as autosomes. They are numbered 1 through 22. In addition, humans have two sex chromosomes, both an X and Y (in males) or two X chromosomes (in females). Thus, the total number of chromosomes is 46 in a normal diploid cell. The genetic information of one set of chromosomes comes from the mother of the individual and the other set from the father. Gametes (i.e., eggs and sperm) are haploid and contain only one set of chromosomes (23 chromosomes in human gametes), so that upon fertilization, a diploid zygote is formed.

38.

B. Most of the lifetime of a cell is spent in G1 phase, during which the cells can produce their specialized proteins and accomplish their essential functions. However, when the signal is received for cell division, the cell enters S phase. In S phase the DNA in all chromosomes is duplicated. At the end of S phase, the duplicated chromosomes remain attached at the centromere. A time delay, G2, separates events of the actual separation of individual chromosomes from their duplicated pairs. Next, the M phase or mitosis is when the two members of each pair of chromosomes go to opposite ends of the original cell. This separates 46 chromosomes into two sets of 23 in each cell. Finally, a cleavage furrow is formed and separates the original cell into two daughter cells. Each cell contains a copy of all the genetic information from each parent.

39.

C. Purified DNA is relatively stable provided it is reconstituted in buffer that does not contain DNases. Therefore, high-quality reagents and type I sterile water should be used in preparing buffers used for this purpose. Experiments have shown that purified DNA is stable for as long as 3 years at refrigerated temperature (4°C). However, long-term storage of purified DNA is best accomplished at –20 to –70°C in a freezer that is not frost free to avoid freeze-thaw cycles that may damage DNA and by dividing the original DNA sample into multiple small aliquots for storage.

40.

C. Refer to Color Plate 57■. Given that the probe used will recognize the trinucleotide repeat found in the fragile X gene, *FMR-1,* the location of positive signals will give information about the size of the repeat sequence within each person's DNA. The normal allele for *FMR-1* has 6–50 trinucleotide repeats (found in normal individuals), the premutation for *FMR-1* contains 50–200 trinucleotide repeats (found in unaffected individuals), and the disease allele (found in affected individuals) has >200 repeats. Because electrophoresis separates DNA by size such that the larger fragments travel shorter distances than smaller fragments, then the larger fragment in the affected individual caused by the expansion of the trinucleotide repeat would be represented in Color Plate 57■ by lane 4 of the diagram.

41.

B. Amplification methods can be automated and standardized, which is proven by the variety of test systems presently on the market. Amplification methods are very sensitive and theoretically can detect one target DNA molecule in a sample. However, increased sensitivity raises the likelihood of false positive results due to contamination of testing areas with PCR amplicons. In addition, most amplification methods can be completed within 4–6 hours and can detect microorganisms that do not grow readily by standard culture techniques. At this time, test reagents are still quite expensive, although if decreased turn-around time would translate into shorter hospital stays, then resultant healthcare costs could be reduced by use of these methods in the clinical laboratory. A disadvantage of amplification technologies is that they require a unique set of primers for each target DNA being sought. Thus, amplification techniques may be replaced by use of DNA microarrays because thousands of genes can be assessed at one time, rather than a limited number of molecules of interest being assayed.

42.

A. Reverse transcription–polymerase chain reaction (RT-PCR) is used to detect gene expression; genes are expressed by transcription into mRNA. The starting material for RT-PCR is mRNA. The only method listed whose target sequence is found in mRNA is RT-PCR. Transcription mediated amplification targets are usually ribosomal RNA. In ribotyping, rRNA probes detect ribosomal RNA genes present in total bacterial DNA; bacteria can be grouped on the basis of banding patterns that result. Multiplex PCR describes a method in which DNA is the target or template, and several different primer sets are included in the reaction mix. An example of multiplex PCR would include methods that detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in one reaction mix.

43.

D. Hepatitis C virus (HCV) has an RNA genome, and thus a reverse transcription step is needed to convert RNA into complementary DNA for use in the subsequent PCR that makes multiple copies of the target sequence. RT-PCR is both highly specific and sensitive. Viral load testing also requires that the methodology be quantitative. Quantification can be accomplished by qPCR techniques or by inclusion of a known amount of a synthetic nucleic acid, a quantification standard (QS), in the sample. The QS binds the same primers as the viral target, and so the kinetics of amplification for both may be assumed to be approximately equal. The viral target and QS are coamplified in the same reaction, and the raw data are manipulated mathematically to determine the viral load present in the specimen. To detect genetic sequences specific for the human leukocyte antigen (HLA) loci, bacteria, and gene mutations, the starting material is usually DNA; therefore, PCR methods, rather than RT-PCR, would be employed.

44.

A. The sensitivity of amplification techniques can be viewed as a double-edged sword. On one hand, the techniques have allowed detection of genetic sequences that are found in limited numbers within a sample. However, because the method creates large amounts of target sequence, the areas within the laboratory can become contaminated with amplicons. Amplicon contamination produces false positive results. The use of dUTP in the reaction mix results in PCR products (i.e., amplicons) containing uracil in place of thymidine. The enzyme used to decrease contamination of previously generated dU-containing amplicons is uracil-*N*-glycosylase (UNG). Samples are pretreated with this enzyme before their use in subsequent PCR reactions to remove contaminating dU-containing amplicons if present. Pretreatment with UNG has no effect on sample DNA containing thymidine residues. Other procedures necessary to avoid contamination include dedicated areas for reagent preparation, impeccable technique, amplification and post-amplification analysis, and use of aerosol-barrier pipette tips. Treatment of work surfaces, equipment, and pipettors with UV light can also be used to prevent contamination.

45–47.

(45:D, 46:B, 47:C)

Questions 45–47 are associated with Color Plates 58a■ and b■. The reaction depicted in Color Plates 58a■ and b■ is the polymerase chain reaction (PCR). It is the prototype of target amplification methods. Other target amplification methods include transcription-mediated amplification (TMA) and nucleic acid sequence–based analysis (NASBA). Traditional PCR is the *in vitro* equivalent of DNA replication *in vivo*. The components of PCR are a DNA template (containing target sequence of interest), a set of oligonucleotide primers (that flank the region of interest), building blocks of deoxynucleotide triphosphates (dATP, dCTP, dGTP, TTP, collectively referred to as dNTPs), and a heat-stable DNA polymerase (e.g., *Taq* polymerase) and buffer providing optimal conditions for primer annealing and DNA synthesis. Optimal salt concentrations (e.g., KCl) are needed for annealing of primers. In addition, optimal concentrations of the divalent cation Mg^{2+}, a cofactor for *Taq* polymerase that determines the fidelity of DNA replication *in vitro,* are essential. PCR consists of cycles of three steps: (1) denaturation of ds template, (2) annealing of ss oligonucleotide primers to complementary sequence in denatured template, and (3) DNA synthesis catalyzed by *Taq* polymerase. During synthesis, primers are extended in the 5' → 3' direction, adding adenine, guanine, cytosine, and thymidine nucleotide bases into the growing chain according to the complementary sequence to which it is bound. The steps generally occur at the following temperatures and duration: denaturation ≥ 94°C (20–60 seconds); annealing (dependent upon base sequences of primers) between 55–65°C (20–90 seconds); and DNA synthesis at 72°C, the optimal temperature for *Taq* polymerase (10–60 seconds). The resulting cDNA products are called amplicons. Starting with one dsDNA molecule (1A:1B) containing a target sequence, denaturation separates the dsDNA template into two ssDNA strands (1A and 1B). Next, the temperature is lowered to a point where specific hybridization of each primer to its complementary sequence in the denatured strands occurs. Then, the temperature is increased to 72°C, and extension of the primers ensues. At the end of cycle #1, two dsDNA parent (1) daughter (2) hybrid molecules result (i.e., 1A:2B and 1B:2A). At the end of cycle #2, four dsDNA products are produced. Intermediate-length products, larger than the target sequence of interest (4A and 4B length) but smaller than the original DNA template (1A:1B), are present. In subsequent cycles, the precise length target sequence is amplified most efficiently and becomes the preferred target for amplification. At each cycle, the number of copies doubles such that after N doublings, 2^{N-2}, copies of target are produced. For example, after 30 cycles, 2^{28} amplicons = 2.68×10^8 (over a billion) copies are made.

In transcription-mediated amplification methods, such as TMA and NASBA, starting material and primary products are both RNA. Enzymes used in these methods are currently avian myeloblastosis virus (AMV) reverse transcriptase with inherent RNase H activity and RNA polymerase (e.g., T7 RNA polymerase). Advantages of TMA and NASBA are that (1) they are isothermal (i.e., do not require a thermocycler); and (2) they target RNA for direct detection of RNA viruses, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV), or rRNA of bacteria, resulting in increased sensitivity. In addition to target amplification, there are other amplification methods in which either probe or signals are amplified. Ligase chain reaction, strand displacement amplification, and Q-beta replicase are examples of probe amplification systems. Signal amplification includes methods such as a hybrid capture assay for human papillomavirus; branched chain DNA methods used for viral load assays for HCV and HIV-1; cleavage-based amplification primarily for factor V Leiden mutation detection; and the cycling probe method of amplification, which has been used to detect genes associated with antimicrobial resistance in bacteria, such as *mec*A in *Staphylococcus aureus* and *van*A and *van*B in *Enterococcus*.

48.

C. Refer to Color Plate 59■. Factor V Leiden mutation (A506G) causes activated protein C resistance that results in increased risk of hypercoagulability. The mutation destroys a *MnlI* restriction enzyme site in an amplified 223 bp PCR product from patient DNA. From the electrophoretic pattern, wild-type or normal factor V will show three bands after *MnlI* digestion (104 bp, 82 bp, 37 bp), as in patients 1, 4, and 5. The pattern seen with patient 2 is that of a homozygous mutant with two bands (141 bp and 82 bp). In the heterozygous patient 3, one allele is normal and the other is mutant. Thus, the banding pattern results in four bands (141 bp, 104 bp, 82 bp, and 37 bp). Sometimes the 37 bp fragment band is not seen because it is below detectable levels.

49.

D. The translocation resulting in the Philadelphia chromosome can be detected by reverse transcription–polymerase chain reaction (RT-PCR), Southern blot, and cytogenetic analysis. The presence of a Philadelphia (Ph) chromosome confirms the diagnosis of chronic myelogenous leukemia (CML). The Ph chromosome is a shortened chromosome 22 that arises from a reciprocal translocation involving the long arms of chromosomes 9 and 22. This translocation involves the proto-oncogene c-*ABL,* normally present on chromosome 9q34, and the *BCR* gene on chromosome 22q11. The juxtaposition of *ABL* with *BCR* results in the formation of a *BCR-ABL* fusion gene, which is subsequently transcribed into a chimeric BCR-ABL mRNA that is ultimately translated into a chimeric BCR-ABL protein product. Traditionally, this rearrangement can be seen cytogenetically by visualization of the patient's karyotype (i.e., metaphase spread of patient's chromosomes). Recent techniques have been developed in which fluorescent-labeled probes for this gene rearrangement can be used to probe the patient's metaphase or interphase spread, called fluorescence *in situ* hybridization (FISH). Molecular methods to check for this gene rearrangement include Southern blotting and RT-PCR. PCR cannot be used for this particular gene rearrangement because BCR/*abl* breakpoints span large segments of DNA, which prevents direct PCR testing. Instead, RT-PCR is used. The BCR/*abl* chimeric mRNA is used as a template because primer annealing sites in the breakpoint region of the mRNA are a smaller size, suitable for amplification.

50.

B. Color Plate 60■ is graphic display of a real-time PCR (i.e., qPCR) run for cytomegalovirus (CMV). Real-time PCR assays can measure the amount of starting target sequence (i.e., template in sample) accurately. Rather than measuring PCR product generated at the stationary or end-point of the PCR assay, qPCR analysis is done as PCR products are formed (i.e., during the exponential phase) where accumulation of fluorescence is inversely proportional to the amount of starting template (i.e., the shorter the time to accumulate signal, the more starting material). Optimal threshold level is based on the background or baseline fluorescence and the peak fluorescence in the reaction and is automatically determined by the instrument. Using 10-fold dilutions of known positive standards, a standard curve can be made. The qPCR cycle at which sample fluorescence crosses the threshold is the threshold cycle (C_T). Using the standard curve, the starting amount of target sequence in each sample can be determined by its C_T. Fluorescence versus C_T is an inverse relationship. The more starting material, the fewer cycles it takes to reach the fluorescence threshold (i.e., large amounts of fluorescence accumulate in a short time). The C_T for sample 5 is 21, sample 4 is 25, and sample 11 is 38; therefore, sample 5 has more CMV copies than sample 4, which has more CMV copies than all the other samples with C_T values indicated, including sample 11 with the least CMV. The samples below the threshold fluorescence of 30 are negative for CMV.

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Farkas, D. H. (2004). *DNA from A to Z.* Washington, DC: AACC Press.

INTERNET REFERENCES

Following is a listing of techniques and the manufacturer that presently owns the technology. This information is included so you can search manufacturers' Web sites to obtain the most current information regarding technologies now being used and/or developed.

The polymerase chain reaction rights were purchased by Hoffman-LaRoche and are now marketed by Roche Diagnostics as the AMPLICOR® product line (http://us.labsystems. roche.com and http://www.mylabonline.com/products/molecular.php).

Transcription-mediated amplification and hybridization protection assay were developed by Gen-Probe, Inc. (http://www.gen-probe.com/sci_tech/tma.htm).

Strand displacement amplification is a product of Becton-Dickinson Biosciences under the product name BDProbeTec™ (http://www.bd.com/ds/technicalCenter/whitepapers/LR598.pdf).

Nucleic acid sequence–based analysis, now called NucliSens®, is being developed and distributed by Organon Teknika (http://www.biomerieux-diagnostics.com/).

Branched chain DNA is a technology developed by Chiron Diagnostics, acquired by Bayer Corporation, and now part of Siemens Medical Solutions (http://diagnostics.siemens.com and http://www.fda.gov/cber/PMAsumm/P0000280S.pdf).

Hybrid capture® assays were developed and are marketed through Digene Corporation (http://www.digene.com/labs/labs_hybrid.html).

Invader® assays based on activity of the Cleavase enzyme were developed and are marketed by Third Wave Technologies, Inc. (http://www.twt.com/invader/invader.html).

Molecular Beacons techonology is available through Public Health Research Institute Properties, Inc. (PHRI). PHRI offers nonexclusive worldwide licenses for the Molecular Beacons technology (http://www.molecular-beacons.org/Introduction.html) and for instruments compatible with the technology (http://www.molecular-beacons.org/PA_instr.html).

The Scorpion® technique was developed by DxS Ltd. (http://www.premierbiosoft.com/tech_notes/Scorpion.html).

TaqMan® technologies were developed and are distributed by Applied Biosystems (http://www3.appliedbiosystems.com/AB_Home/index.htm).